# Deletion of the core-*H* region in mice abolishes the expression of three proximal odorant receptor genes in *cis*

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**We have previously reported that a 2.1-kb homology (*H*) sequence, conserved between mouse and human, regulates the odorant receptor (OR) gene *MOR28* in transgenic mice. Here, we narrowed down the essential sequences of the *H* to a core of 124 bp by using a transient expression system in zebrafish embryos. Transgenic experiments in mice demonstrated that the core-*H* sequence is sufficient to endow expression of the *MOR28* minigene. Deletion and mutation analyses of the core-*H* region revealed two homeodomain sequences to be essential for the *H* enhancer activity. Targeted deletion of the core-*H* abolished expression of three proximal OR genes, *MOR28*, *MOR10*, and *MOR83*, in *cis*, indicating the presence of another locus control region/enhancer in the downstream region, that regulates four distal OR genes in the same *MOR28* cluster. In the heterozygous mice, the $H^-$ phenotype of the mutant allele was not rescued by the wild-type $H^+$ allele in *trans*.**

olfactory sensory neuron | gene targeting | locus control region | transgenic mouse | zebrafish

In the mouse olfactory system, there are >1,000 odorant receptor (OR) genes forming the largest multigene family in the genome (1). It is well established that each olfactory sensory neuron (OSN) expresses only one functional OR gene in a mutually exclusive and mono-allelic manner (2–4). Furthermore, OSNs expressing the same OR gene converge their axons to a specific set of glomeruli in the olfactory bulb (OB) (5–7). Thus, the odorant stimuli received in the olfactory epithelium (OE) are converted to a topographic map of activated glomeruli in the OB. How is the one neuron–one receptor rule (8, 9) ensured to form the genetic basis for the OR-instructed axonal projection? Results from mice cloned by nuclear transfer of mature OSNs ruled out models of DNA recombination and gene conversion (10, 11).

We have previously reported a *cis*-acting DNA region that regulates the expression of an OR gene cluster in transgenic mice. Sequence comparison of the mouse and human genomes revealed a 2.1-kb homology (*H*) region, ≈60 kb upstream of the *MOR28* cluster on chromosome 14 of the C57BL/6 mouse (9, 12, 13). Using the yeast artificial chromosome (YAC), we demonstrated that *H* is required for expression of all four OR genes contained within the YAC construct (9). Importantly, this phenotype was not rescued by the endogenous *H* alleles in *trans*. Attachment of the *H* to the *H*-deleted YAC constructs restored expression of all OR transgenes from the YAC. Furthermore, when the *H* region was relocated closer to the cluster, the number of OSNs expressing the proximal OR gene was greatly increased. These results indicated that the *H* region is a *cis*-acting locus control region (LCR)/enhancer that activates the *MOR28* cluster, at least in the transgenic construct. Like other LCRs in the globin gene and the photopigment gene systems in the human (14, 15), we have postulated that a chromatin-remodeling complex assembled at *H* activates one OR gene at a time, by physical association with one promoter site (16).

Recently, Lomvardas *et al.* (17) reported that the *H* sequence can associate with OR promoters in *trans* on nonhomologous chromosomes, like the IL-4 LCR in T helper cells (18). Based on FISH and chromosome conformation capture (3C) analyses of OSN nuclei, they proposed that the *H* acts not only in *cis* but also in *trans*, and that a single *trans*-acting *H* enhancer may allow the stochastic activation of only one OR gene in each OSN. In the present study, we narrowed down and identified the essential sequences in the *H* region. Deletion of the core-*H* sequence in knockout mice abolished expression of three proximal OR genes in the *MOR28* cluster in *cis* from the same allele.

## Results

**Narrowing Down the Essential Sequences of the Mouse *H* Region in Zebrafish Embryos.** The *H* region was identified as a LCR/enhancer that regulates the *MOR28* cluster in transgenic mice (9) (Fig. 1*A*). To narrow down and identify the essential regulatory sequences in *H*, we developed a strategy with zebrafish embryos as an assay for transient expression. We modified a zebrafish OR minigene (*ZOR111-1*) by inserting an *EYFP* tag immediately 3′ to the OR coding region (Fig. 1*B Left*). The parameter for regulatory activity is the percentage of zebrafish embryos that contain at least one fluorescent OSN. Our assay saturated at ≈50–60% of injected embryos exhibiting fluorescence in some of their OSNs. The zebrafish OR gene was not robustly expressed in a minigene form, possibly because of the absence of the LCR/enhancer (19). Minigene expression was greatly enhanced in zebrafish OSNs when the mouse *H* region was attached to the 5′ end in either orientation (Fig. 1 *B* and *C*). The *H* also activated the zebrafish minigene when it was attached to the 3′ end. Interestingly, *H* could not act on the minigene in *trans* from a separate plasmid (Fig. 1*C*). Duplicated *H* did not further increase the frequency of the minigene activation.

We then generated truncated *H* segments of various lengths and examined their activities [Fig. 2*A* and supporting information (SI) Fig. 7]. We narrowed down the functional sequences to a contiguous segment of 124 bp, which we termed the core-*H* region. No additional elements that partially or quantitatively affect the reporter minigene expression were identified outside of the *H*. To pinpoint the binding motifs for nuclear factors, we performed a homology search that revealed three potential homeodomain motifs, ATTAATG (#1 at position 15), TATAATG (#2 at position 32), and CATTAAG (#3 at position 102) in the 124-bp *H* region (Fig. 2*C*). When these motifs were mutated individually by substituting two nucleotides, mutations in #1 and #3 motifs but not in #2 abolished the *H* activity (Fig. 2*B*). We converted every block of 10 bp to a stretch of As between positions 30 and 98 and tested their

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**Fig. 1.** The *H* region enhances OR gene expression in zebrafish. (*A*) Genomic maps of the *MOR28* cluster and surrounding regions on mouse chromosome 14. The cluster contains three ventral-zone OR genes (*MOR28*, *MOR10*, and *MOR83*) and four dorsal-zone OR genes (*MOR29A*, *MOR29B*, *MOR30A*, and *MOR30B*). The *H* region was previously identified as a 2.1-kb *H* region that is conserved between mouse and human. (*B*) A zebrafish OR minigene, *ZOR111-1*, was tagged with *EYFP* by direct fusion to the 3′ end of the coding region. The minigene did not express by itself, possibly because of the absence of the LCR/enhancer. Addition of the mouse 2.1-kb *H* region affords expression of the *ZOR111-1* minigene. Whole mounts of fishes (frontal views) are shown under fluorescent illumination. (Scale bars: 20 μm.) (*C*) (*Left*) Positional effects of the mouse *H* region in zebrafish. (*Right*) The percentages of injected embryos that turned out to be EYFP-positive are shown. The assay saturated at 50–60% of injected embryos. The *H* region acts in *cis* regardless of its direction or location, but does not act in *trans* between plasmids. All assays were performed in triplicates with <20% variance among experiments. Standard deviations are indicated by error bars.

effects on the *H* activity (Fig. 2*C*). Only one other motif was detected between the two homeodomain sequences (#1 and #3), an O/E-like sequence at position 91, which was found to be required for expression in zebrafish.

**The Core-*H* Sequence Is Functional in Transgenic Mice with OR Minigene.** To verify the results obtained with the zebrafish minigene in zebrafish embryos, we generated stable lines of transgenic mice with mouse OR minigenes with or without the core-*H* sequence. First, we examined whether the core-*H* region is indeed functional in mice. We attached the 187-bp sequence containing core-*H* to the *MOR28* minigene tagged with *IRES-gap-EYFP* (Fig. 3*A*). It should be noted that the *MOR28* minigene is not expressed on its own without *H* (9). When the core-*H* sequence was attached, most transgenic lines showed expression, and often a large number of OSNs expressed the *MOR28* minigene (Fig. 3*B*). As with the 187-bp sequence, the 124-bp core-*H* also increased numbers of OSNs that express the *MOR28* minigene as the entire *H* region of 2.1 kb did (SI Table 1). To examine whether the one neuron–one receptor rule is maintained in the minigene-expressing OSNs, coexpression was analyzed by two color *in situ* hybridization (ref. 9 and unpublished observation). In all transgenic lines, no endogenous OR genes were coexpressed with the transgenic *MOR28* in individual OSNs so far examined. In the mouse lines with abundant expression, fluorescence examination of whole mounts revealed a cluster of transgene-positive glomeruli in the OB, rather than a single, enlarged glomerulus (Fig. 3 *C* and *D*). We assume that respective glomeruli represent different expression zones and expression levels of the transgenic *MOR28* that are expressed in the broader area in the OE in various amounts. It has been reported that the dorsoventral arrangement of glomeruli is affected by the anatomical locations of OSNs in the OE (20) and that OR expression levels can affect OSN projection along the anteroposterior axis in the OB (21).

We then mutated the homeodomain sequences, #1 and #3, in the 187-bp core with two nucleotide substitutions. No transgene-positive OSNs or glomeruli were found in all five transgenic lines examined (Fig. 3). We also analyzed strains of transgenic mice carrying a BAC that contains the differently tagged *MOR28*, *MOR10*, and *MOR83*. Expression of these OR genes was greatly reduced when the O/E-homeodomain (#3) region of 21 bp was deleted in the core sequence (SI Fig. 8). Another deletion outside of the core-*H* region, just upstream of the homeodomain sequence #1, did not abolish the transgenic expression. These mouse experiments with the *MOR28* minigenes and BAC transgenes confirmed the results obtained with zebrafish embryos: the core-*H* sequence is sufficient to activate the *MOR28* minigene, and the two predicted homeodomain sequences are indeed functional and necessary for *H*-enhancer activity.

**Knocking Out the Core-*H* Sequence by Homologous Recombination.** We have previously reported that deletion of *H* in a YAC transgene abolishes expression of all four OR genes that reside on this construct (9). Two endogenous alleles of *H* were unable to rescue the expression of OR genes from the YAC, by acting in *trans* across chromosomes (9). To analyze the *in vivo* function of endogenous *H* within its normal configuration, we generated knockout mice lacking the core-*H* sequence (Fig. 4*A*). Using homologous recombination, the core-*H* was mutated by insertion of the neomycin resistance (*neo$^r$*) gene in the ES cells (line D3). After transfection of the targeting vector, >400 colonies resistant to both G418 and ganciclovir were screened by PCR and Southern blot analyses. Three ES clones carrying the mutant allele were isolated and used to generate heterozygous mice. By crossing these mice with transgenic mice that express flippase recombinase, the *neo$^r$* cassette was removed. Both heterozygous and homozygous littermates were healthy and fertile under conventional conditions and did not show anatomical abnormalities in either the OE or the OB.

**Fig. 2.** Dissection of the essential sequences of the mouse *H* region in zebrafish. (*A*) *H* activities of various truncated segments. By comparing the activities in the transient expression assay in zebrafish and lengths of the segments, the essential region was narrowed down to 124 bp. Activities were measured as described for Fig. 1*C*. (*B*) Mutational analysis of the homeodomain sequences. Three potential homeodomain sequences were mutated independently as shown in *C*. Mutations in the two homeodomain sequences at positions 15 (#1) and 102 (#3) abolished the *H* activity. The homeodomain motif at position 32 (#2) was not required for the *H* activity in this assay. Standard deviations are indicated by error bars. (*C*) Putative binding motifs in the core-*H* sequence. Three potential homeodomain motifs (#1, #2, and #3) were found. Replacement of each block of 10 bp with a stretch of As revealed one additional motif, an O/E-like sequence at position 91. Activities are shown in percentages of EYFP-positive zebrafish embryos.



**OR Gene Expression in the Core-*H* Knockout Mice.** We examined the expression of various OR genes in the knockout mice by RT-PCR and *in situ* hybridization of OE sections. RNA could readily be detected for all tested OR genes, except for *MOR28*, *MOR10*, and *MOR83*. *In situ* hybridization of OE sections confirmed this observation. RNA expression of three proximal OR genes in the *MOR28* cluster, *MOR28*, *MOR10*, and *MOR83*, was totally abolished in homozygous ($H^{-/-}$) mutant mice (Fig. 4*B*). In contrast, four distal OR genes in the same cluster, *MOR29A*, *MOR29B*, *MOR30A*, and *MOR30B*, were only partially affected (Fig. 4*B*). No other OR genes, on the same chromosome or on the other chromosomes so far examined, were affected in the core-*H* knockout mice. Vomeronasal receptor genes residing on the same chromosome, e.g., *V2r11*, were expressed normally in the vomeronasal organ.

In heterozygous mutant mice, expression frequencies of *MOR28*, *MOR10*, and *MOR83* were reduced to ≈50%, indicating that the $H^{-}$ phenotype of the mutant allele was not rescued by the wild-type allele in *trans*, between homologous chromosomes (Fig. 4*B*). We also analyzed the expression of two polymorphic *MOR28* alleles in the compound heterozygous mice that carry the *EGFP*-tagged *MOR28* with an intact $H^{+}$ (C57BL/6), and the untagged *MOR28* with a mutant $H^{-}$ (129/SvJ). *MOR28* cDNA was also cloned from heterozygous mice and sequenced after PCR: all clones were derived from the $H^{+}$ (C57BL/6) allele (data not shown). Here again, the *EGFP*-tagged *MOR28* allele ($H^{+}$) did not rescue the mutant allele ($H^{-}$) in *trans*, between homologous chromosomes (Fig. 4*C*).

We have previously reported that deletion of the full 2.1-kb *H*

**Fig. 3.** Functional assays of the core-*H* sequences in transgenic mouse lines. (*A*) Transgenic constructs. A 187-bp segment containing the core-*H* sequence was examined for *H* activity on the *MOR28* minigene, which is not expressed by itself. The mutant segment containing two mutated homeodomain sequences (#1 and #3) was also examined. The *MOR28* minigene was tagged with *IRES-gap-EYFP*. (*B*) Coronal sections of the OE were imaged under fluorescent illumination for EGFP and EYFP. (*Left*) Expression of the endogenous *MOR28* tagged with *IRES-gap-EGFP* (3) is shown. (*Center*) A large number of OSNs expressed the *MOR28* minigene when the 187-bp sequence was attached. (*Right*) When the homeodomain sequences, #1 and #3, were mutated, no transgene-positive OSNs were detected. (*C*) Lateral views of the mouse OB. Glomerular formation for the endogenous *MOR28* (*Left*) and the mutant core-*H* (*Right*) was analyzed as controls. (*Center*) Whole-mount fluorescence revealed groups of the EYFP-positive glomeruli, when the 187-bp core-*H*-containing sequence was attached to the *MOR28* minigene. (*D*) Coronal sections of the OB. (*Upper*) Multiple glomerular structures were found for the *MOR28* minigene with the 187-bp core-*H*-containing sequence. (*Lower*) Higher magnification is also shown. (Scale bars: *B*, 20 μm; *C*, 500 μm; *D*, 50 μm.)

Fig. 4. OR gene expression in core-*H* knockout mice. (*A*) Targeted deletion of the 187-bp core-*H* containing sequence. By homologous recombination between the targeting vector and the endogenous *H* region, the 187-bp sequence containing the core-*H* was replaced with the FRT-*neo*$^r$- FRT cassette. By crossing the mutant mice with transgenic mice (CAG-FLP) that expresses FLP recombinase, the *neo*$^r$ gene was removed. The 2.1-kb *H* region is shown in green and its core sequence is in red. Restriction enzyme sites are: *S*, SphI, and *H*, HindIII. (*B*) *In situ* hybridization of OE sections. Various OR genes and a vomeronasal receptor gene, *V2r11*, were analyzed for their expression at the RNA level by single-color *in situ* hybridization. In homozygous core-*H* knockout mice, RNA for three OR genes in the *MOR28* cluster (*MOR28*, *MOR10*, and *MOR83*) was not detected. The numbers of OSNs expressing these three genes were reduced to approximately half in the heterozygous knockout animal compared with the wild type. Four distal OR genes in the *MOR28* cluster (*MOR29A*, *MOR29B*, *MOR30A*, and *MOR30B*) were partially affected. For quantification of OR gene expression, total numbers of probe-positive OSNs in the OE were counted in three 3-week-old mice. Probe-positive cell numbers were compared between the heterozygous ($H^{+/-}$) or homozygous ($H^{-/-}$) mutant mice and the wild-type ($H^{+/+}$) littermates. Standard deviations are indicated by error bars. (*C*) The *H* in the wild-type allele ($H^+$) does not rescue the *MOR28* expression from the mutant allele ($H^-$) in *trans*. After crossing the *EGFP*-tagged *MOR28* mouse with the *H*-deletion mouse, we analyzed the expression of the *MOR28* gene and the *EGFP* tag by two-color *in situ* hybridization. The intact *H* region of the *EGFP*-tagged wild-type allele ($H^+$) did not rescue the defective phenotype of *MOR28* expression from the mutant allele ($H^-$).

region abolished expression of all four OR genes, *MOR28*, *MOR10*, *MOR83*, and *MOR29A*, in the YAC construct (9). In contrast to the transgenic results, core-*H* deletion in the knockout mice abolished expression of only three *H*-proximal OR genes (*MOR28*, *MOR10*, and *MOR83*), and partially affected four distal OR genes (*MOR29A*, *MOR29B*, *MOR30A*, and *MOR30B*). Basically the same result was obtained with the knockout mice deleting the full 2.1-kb *H* region (21). These observations indicate the presence of another LCR/enhancer downstream of the *MOR28* cluster that would regulate the four distal OR genes (see Fig. 6*A*). It should be noted that the YAC construct lacks the 3′ region of the cluster including *MOR29B*, *MOR30A*, and *MOR30B* (9).

**Multiple Regulatory Elements for a Large OR Gene Cluster in Zebrafish.** We also searched for LCRs/enhancers in zebrafish OR genes. Using the transient expression system with zebrafish embryos, we analyzed three BAC clones that contain OR gene clusters located on the zebrafish chromosome 15 (CH211-244E12 and CH211-133D24) or 21 (CH211-51A20). To visualize the activation of OR promoters, OR coding sequences were replaced with fluorescent marker genes. The *ZOR111-1* and *ZOR116-1* were replaced with *EYFP* and *ECFP*, respectively, in BAC clone CH211-244E12. We then generated various truncated BAC constructs and examined them for transient expression of marker genes in zebrafish embryos. By comparing the expression patterns and structures of the transgenic constructs, we identified two LCRs/enhancers, *E15-1* and *E15-2*, in the same OR gene cluster (Fig. 5*A* and SI Figs. 9 and 10). These LCR/enhancer sequences were not similar to each other and have no obvious homologies to the mouse *H* sequence, except for some homeodomain-like sequences and OE-like sequences in the zebrafish enhancers (GenBank accession nos. AB33167 and AB331638). We then generated truncated *E15-1* and *E15-2* segments of various lengths and examined their enhancer activities to express the *ZOR111-1* minigene. We narrowed down the functional sequences of *E15-1* and *E15-2* to 0.7- and 2.3-kb segments, respectively (SI Fig. 10). The *E15-1* segment also activated the *EYFP*-tagged *ZOR104-2* promoter, when it was attached to the BAC clone (CH211-2154A9) (Fig. 5*B*). A combined deletion of both *E15-1* and *E15-2* abolished expression of two differently labeled OR genes, *ZOR111-1* (*EYFP*) and *ZOR116-1* (*ECFP*) on the BAC (Fig. 5*A*). Interestingly, however, deletion of either of the two LCRs did not abolish OR gene expression from the BAC (Fig. 5*A*). We assume that these two LCRs/enhancers may function in an overlapping manner. Recently, it was reported that OR genes in the BAC clone (100G14) that covers the downstream region of BAC CH211–244E12, are robustly expressed (22). Because BAC 100G14 does not contain the LCRs/enhancers identified in our BAC clone, the third LCR/enhancer may be present in this OR gene cluster. These observations are suggestive of independent but overlapping effects of multiple LCRs/enhancers within an OR-gene cluster (Fig. 6*C*).

## Discussion

**The Core-*H* Sequence Only Acts in *Cis*.** Lomvardas *et al.* (17) reported that the *H* sequence can associate with OR promoters in *trans* on nonhomologous chromosomes, like the *IL-4* LCR in T helper cells (18). Based on FISH and 3C analyses of OSN nuclei, they proposed that the *H* acts not only in *cis* but also in *trans*, and that a single *trans*-acting *H* enhancer may allow the stochastic activation of only one OR gene in each OSN.

Our previous transgenic experiments, however, were not consistent with the *trans*-acting enhancer model: the *H* deletion from the YAC constructs was not complemented by the endogenous *H* (9). In the present study, we generated and analyzed two strains of core-*H* knockout mice. In the heterozygous mice, core-*H* deletion was not rescued by the wild-type *H* on the other allele in *trans*. Deletion of the core-*H* only affected the *MOR28* cluster but not other OR gene clusters. It can be argued that the core-*H* may be necessary and sufficient for the *cis*-enhancer activity, but not necessary for the proposed *trans*-acting function. However, Mombaerts and his colleagues (23) performed knockout experiments for the entire *H* region of 2.1 kb and obtained basically the same results as we did.

Fig. 5. LCRs/enhancers in zebrafish OR genes. (*A*) BAC clone CH211–244E12. Two OR genes, *ZOR111-1* and *ZOR116-1*, were differently labeled by replacing their coding sequences with those for *EYFP* and *ECFP*, respectively. By comparing the expression patterns and truncated versions of the BAC clones, two LCRs/enhancers (*E15-1* and *E15-2*) were identified. Expression rates (%) were measured by counting the EYFP/ECFP-positive embryos as described in Fig. 1*C*. (*B*) Activation of OR genes by the attachment of the *E15-1*. The 0.7-kb *E15-1* segment, isolated from the BAC CH211-244E12, activated the *EYFP*-tagged *ZOR104-2* promoter when it was attached to the BAC CH211-215A9.

**Extent of the Effect of *H* Region Activity.** In our previous study, deletion of *H* in the YAC constructs abolished expression of *MOR28*, *MOR10*, *MOR83*, and *MOR29A* (9). This discrepancy with the knockout phenotype, in which expression of *MOR29A* was only partially affected, may be caused by the absence of a second enhancer on the YAC constructs, which would act on the *MOR29A* and other three distal OR genes. But, how is it that the addition of the *H* to the truncated YAC constructs restored the expression of all four OR transgenes in the *MOR28* cluster, including *MOR29A* (9)? Because multiple YAC constructs integrated into a single chromosomal locus in a tandem fashion, we assume that the *MOR29A* was activated by the neighboring *H* region on the adjacent YAC construct downstream.

As mentioned above, the core-*H* deletion abolished expression of only three *H*-proximal OR genes. It is interesting that effects of the core-*H* deletion on other distal OR genes in the cluster were only partial and roughly inversely proportional to the distances from the *H*. We, therefore, postulate the presence of another LCR/enhancer in the downstream region regulating the distal portion of the cluster (Fig. 6*A*). In zebrafish, hierarchical regulation of OR gene choice was recently reported, using BAC transgenic constructs carrying an OR gene cluster (22). In our present study, at least two enhancer elements, *E15-1* and *E15-2*, were identified in the OR gene cluster (BAC CH211-244E12) in zebrafish (Fig. 6*B* and SI Figs. 9 and 10). These enhancers activate the OR genes not only in a minigene form but also in a BAC clone. It will be interesting to study whether the zebrafish enhancers function like the *H* in the mouse and whether the insulator-like element separates the effects of these enhancers. We postulate that multiple *cis*-acting enhancers regulate the OR genes within a cluster in an independent, but partly overlapping, manner in both the mouse and zebrafish (Fig. 6*C*).

Fig. 6. Regulation of vertebrate OR genes by multiple *cis*-acting LCRs/enhancers. (*A*) Expression of mouse OR genes in the *MOR28* cluster on chromosome 14. The core-*H* knockout abolished expression of just three *H*-proximal OR genes (*MOR28*, *MOR10*, and *MOR83*). There may be another LCR further downstream regulating the distal OR genes (*MOR29A*, *MOR29B*, *MOR30A*, and *MOR30B*). (*B*) Multiple LCRs/enhancers in a zebrafish OR gene cluster. Deletion analyses of BAC clones revealed at least two LCRs/enhancers within the OR gene cluster on zebrafish chromosome 15. (*C*) A model for OR gene activation. OR genes would be regulated by multiple *cis*-acting LCRs/enhancers in an overlapping and partially redundant manner.

**Regulation of OR Gene Expression.** Previous transgenic studies indicated that short and proximal regulatory regions are sufficient to activate OR minigenes (24–26). Curiously, mutations in the factor binding sites affected differently between the transgenic OR gene and the endogenous gene: mutations abolished the transgene expression, however, affected only partially the endogenous gene (26). We assume that the OR minigene did not contain all necessary regulatory regions and that expression of the transgenic minigene was regulated by the LCR/enhancer region, which happened to reside near the transgene in the chromosome.

Mutagenesis of the core-*H* sequence revealed at least two functional homeodomain sequences at positions 15 and 102 in the 124-bp core-*H* region. Homology searches predicted another motif, an O/E-like sequence, at position 91. This sequence was shown to be functional in the zebrafish, although not yet confirmed in mice. Both the homeodomain and O/E-like sequences are often found in the OR promoter regions (24–27). Homeodomain factors, Lhx2 and Emx2, and O/E family proteins are known to bind to their motifs in the OR gene promoter (28, 29). We assume that these nuclear factors bind to *H* and form a complex that remodels the chromatin structure near the cluster, thereby activating one OR promoter site in *cis* at a time by physical interaction. This model is attractive because it reduces the likelihood of the simultaneous activation of multiple OR genes, from a probability among ≈1,400 genes to that among ≈100 LCR/enhancer loci.

In the retina, an LCR plays an important role in choosing either the red or green photopigment gene in a mutually exclusive manner in cone cells (15, 30). It is assumed that stochastic interaction between the LCR and either of the two promoters ensures mutually exclusive expression of the human photopigment genes from the X chromosome. In the OR gene system, the LCR–promoter interaction alone would not preclude the activation of a second OR gene from other OR gene clusters in the genome. To maintain the one

neuron–one receptor rule in OSNs, negative feedback regulation may be taking place with functional ORs (9, 31). It is known that a substantial number of pseudogenes are present in the mammalian OR gene families (≈30% of total OR genes in the mouse and ≈60% in human are nonfunctional). If an activated LCR has selected a pseudogene and has been trapped by its promoter, other LCRs must undergo a similar process to ensure the activation of an intact OR gene (32). Pseudogene may help slow down the process of OR gene activation and further reduce the frequency of the simultaneous activation of two different OR genes. Although the exact mechanisms are yet to be defined, we propose that multiple *cis*-acting LCRs, together with negative feedback by functional OR molecules, ensure the establishment and maintenance of the one neuron–one receptor rule in the mouse olfactory epithelium.

## Materials and Methods

**Zebrafish Experiments.** BAC clones containing zebrafish OR gene clusters were obtained from the BACPAC Resources Center at Children's Hospital Oakland Research Institute, Oakland, CA. The nomenclature for zebrafish OR genes (33) was adopted in this article. The zebrafish OR minigene *ZOR111-1* was isolated from a BAC clone (CH211-244E2) with KpnI and subcloned into the KpnI site of pBlueScriptII vector (Stratagene). The 5′ and 3′ franking sequences are 1.7 and 7.3 kb long, respectively. The *EYFP* tag was inserted and fused to the *ZOR111-1* coding sequence in-frame to produce an OR-EYFP fusion protein. Various *H*-region sequences were inserted into the SacII–XbaI cloning site in the vector. Mutagenesis in the core-*H* region was performed with a QuikChange site-directed mutagenesis kit (Stratagene). BAC modification was performed with a CounterSelection BAC Modification Kit (Gene Bridges). To visualize the activation of OR promoters, OR coding sequences were replaced with *EYFP* or *ECFP*.

Zebrafish embryos were incubated at 28°C in embryo medium (EM), containing 17 mM NaCl, 0.27 mM $CaCl_2$, 0.66 mM $MgSO_4$, 0.4 mM KCl, and $1 \times 10^{-5}$% methylene blue. For microinjection, DNA was linearized and dissolved (50–100 ng/μl) in 100 mM KCl, containing 0.05% phenol red for injection (0.2–0.5 nl) into the cytoplasm of the one- to two-cell embryos. Dead embryos and unfertilized eggs were removed several hours after microinjection. The total number of embryos was scored and expressed as percentage of surviving embryos (penetrance). The expressivity was determined as number of positive OSNs per positive olfactory placode. Zebrafish embryos were screened with fluorescent microscopy for the expression of the OR minigene in OSNs 24–72 h after fertilization. Embryos were embeded in 1% low-melting point agarose in EM containing 0.02% 3-aminobenzoic acid ethyl ester (tricaine; Sigma) and imaged by confocal microscopy (Olympus).

**Generation of the Core-*H* Sequence Knockout Mouse.** Segments of 2.1 kb of the 5′ flanking region and 4.1 kb of the 3′ flanking region of the 187-bp core-*H* region were cloned from C57BL/6-derived BAC DNA by PCR. Segments were inserted into the NotI–XhoI and PacI–KpnI sites in a pNT1.1 vector to generate the targeting vector, which was linearized and introduced into ES cells of line D3, which is of 129/SvEv origin. ES cell clones resistant to both G418 and ganciclovir were screened for homologous recombination by PCR. Three recombinant clones were found among 432 resistant clones and injected into C57BL/6 blastocysts. Chimeric mice were mated with C57BL/6 mice, and the resulting heterozygous offsprings were crossed with CAG-FLP transgenic mouse (C57BL/6 background) (34) to remove the *neo*$^r$ gene. Screening primers were genome, *TGTTCCCTAAGATTTTCTACATCAGAGTCACGAGC*; neo, *GCCTTCTATCGCCTTCTTGACGAGTTCTTC*. Genotyping primers were: HF, *CTAATTTACTTACATGAATTATTCACCTTGCAACT*; HR, *CAGGGATTATCTGATAAGATGAACAACCCA*.

**Histological Analysis of Olfactory Tissue.** All animal experiments were carried out in accordance with guidelines at the University of Tokyo. All efforts were made to minimize the number of animals used. Mice were anesthetized with sodium pentobarbital (2.5 mg/animal) and perfused intracardially with 4% paraformaldehyde in PBS. OE and OB were dissected and fixed overnight with 4% paraformaldehyde in PBS. The OE was decalcified in 0.5 M EDTA at 4°C for 1–2 days. Tissues were then placed in 30% sucrose and embedded quickly in OCT compound (Tissue-Tek) in liquid nitrogen vapor. Serial coronal sections (12 μm for the OE; 16 μm for the OB) were prepared with a JUNG CM3000 cryostat (Leica) and mounted onto glass slides coated with 3-aminopropyl-triethoxysilane. For *in situ* hybridization, digoxigenin-labeled probes for OR genes were prepared with a DIG RNA labeling kit (Roche). To analyze expression of OR genes, coronal sections of the OE (12 μm each) from 3-week-old mice were hybridized with the probes as described (20, 35) (SI Table 2). Probe-positive OSNs were counted under an Olympus AX70 microscope.

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1. Buck L, Axel R (1991) *Cell* 65:175–187.
2. Chess A, Simon I, Cedar H, Axel R (1994) *Cell* 78:823–834.
3. Serizawa S, Ishii T, Nakatani H, Tsuboi A, Nagawa F, Asano M, Sudo K, Sakagami J, Sakano H, Ijiri T, *et al.* (2000) *Nat Neurosci* 3:687–693.
4. Ishii T, Serizawa S, Kohda A, Nakatani H, Shiroishi T, Okumura K, Iwakura Y, Nagawa F, Tsuboi A, Sakano H (2001) *Genes Cells* 6:71–78.
5. Ressler KJ, Sulivan SL, Buck LB (1994) *Cell* 79:1245–1255.
6. Vassar R, Chao SK, Sitcheran R, Nunez JM, Vosshall LB, Axel R (1994) *Cell* 79:981–991.
7. Mombaerts P, Wang F, Dulac C, Chao SK, Nemes A, Mendelsohn M, Edmondson J, Axel R (1996) *Cell* 87:675–686.
8. Malnic B, Hirono J, Sato T, Buck LB (1999) *Cell* 96:713–723.
9. Serizawa S, Miyamichi K, Nakatani H, Suzuki M, Saito M, Yoshihara Y, Sakano H (2003) *Science* 302:2088–2094.
10. Eggan K, Baldwin K, Tackett M, Osborne J, Gogos J, Chess A, Axel R, Jaenisch R (2004) *Nature* 428:44–49.
11. Li J, Ishii T, Feinstein P, Mombaerts P (2004) *Nature* 428:393–399.
12. Nagawa F, Yoshihara S, Tsuboi A, Serizawa S, Itoh K, Sakano H (2002) *Gene* 292:73–80.
13. Lane RP, Roach JC, Lee IY, Boysen C, Smit A, Trask BJ, Hood L (2002) *Genome Res* 12:81–87.
14. Li Q, Harju S, Peterson KR (1999) *Trends Genet* 15:403–408.
15. Smallwood PM, Wang Y, Nathans J (2002) *Proc Natl Acad Sci USA* 99:1008–1011.
16. Serizawa S, Miyamichi K, Sakano H (2004) *Trends Genet* 20:648–653.
17. Lomvardas S, Barnea G, Pisapia D, Mendelsohn M, Kirkland J, Axel R (2006) *Cell* 126:403–413.
18. Spilianakis CG, Lalioti MD, Town T, Lee GR, Flavell RA (2005) *Nature* 435:637–645.
19. Mori T, Sakai M, Matsuoka I, Kurihara K (2000) *Biol Pharm Bull* 23:165–173.
20. Miyamichi K, Serizawa S, Kimura HM, Sakano H (2005) *J Neurosci* 25:3586–3592.
21. Imai T, Suzuki M, Sakano H (2006) *Science* 314:657–661.
22. Sato Y, Miyasaka N, Yoshihara Y (2007) *J Neurosci* 27:1606–1615.
23. Fuss SH, Omura F, Mombaerts P (2007) *Cell* 130:373–384.
24. Vassalli A, Rothman A, Feinstein P, Zapotocky M, Mombaerts P (2002) *Neuron* 35:681–696.
25. Hirota J Mombaerts P (2004) *Proc Natl Acad Sci USA* 101:8751–8755.
26. Rothman A, Feinstein P, Hirota J, Mombaerts P (2005) *Mol Cell Neurosci* 28:535–546.
27. Michaloski JS, Galante PA, Malnic B (2006) *Genome Res* 16:1091–1098.
28. Wang SS, Lewcock JW, Feinstein P, Mombaerts P, Reed RR (2004) *Development* 131:1377–1388.
29. Hirota J, Omura M, Mombaerts P (2007) *Mol Cell Neurosci* 34:679–688.
30. Wang Y, Smallwood PM, Cowan M, Blesh D, Lawler A, Nathans J (1999) *Proc Natl Acad Sci USA* 96:5251–5256.
31. Lewcock JW, Reed RR (2003) *Science* 302:2078–2079.
32. Roppolo D, Vollery S, Kan CD, Luscher C, Broillet MC, Rodriguez I (2007) *EMBO J* 26:3423–3430.
33. Alioto TS, Ngai J (2005) *BMC Genomics* 6:173.
34. Schaft J, Ashery-Padan R, van der Hoeven F, Gruss P, Stewart AF (2001) *Genesis* 31:6–10.
35. Tsuboi A, Yoshihara S, Yamazaki N, Kasai H, Asai-Tsuboi H, Komatsu M, Serizawa S, Ishii T, Matsuda Y, Nagawa F, *et al.* (1999) *J Neurosci* 19:8409–8418.